SUPPRESSION OF MANGANESE-DEPENDENT PRODUCTION OF NITRIC OXIDE IN ASTROCYTES: IMPLICATIONS FOR THERAPEUTIC MODULATION OF GLIAL-DERIVED INFLAMMATORY MEDIATORS

A Thesis

by

TYLER T WRIGHT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

December 2006

Major Subject: Biology

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ABSTRACT

Suppression of Manganese-Dependent Production of Nitric Oxide in Astrocytes: Implications for Therapeutic Modulation of Glial-Derived Inflammatory Mediators.

(December 2006)

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Primary cultured astrocytes were treated with Mn in the absence and presence of pro-inflammatory cytokines to determine their effect upon stimulation of nitric oxide (NO) production. Treatments of manganese and cytokines raised NO production to intermediate levels, whereas combined treatment raised NO creation to much greater levels. Furthermore, this combined treatment differed from control only in its ability to elevate cellular NO levels at 24 hours, but not at earlier time points. Combined exposure in astrocytes derived from mice lacking the *nos2* gene prevented any increase in production of NO. Thus, manganese and cytokines enhance NO production through activation of the *nos2* gene. Additionally, pharmacologic ligands of the peroxisome proliferator-activated receptor gamma (PPARγ) were used to test the role of this orphan nuclear receptor in modulating Mn-dependent production of NO. The agonist, 1,1-Bis(3'-indolyl)-1-(p-trifluormethylphenyl) methane (cDIM1) diminished NO in a dose-dependent manner, whereas addition of the PPARγ antagonist, GW 9662, amplified cellular NO production, also in a dose-dependent fashion. Moreover, it was observed that NO production was both attenuated and augmented at similar

rates, suggesting the agonist and antagonist work through similar mechanisms. To clarify the means by which NO levels are manipulated by PPARγ, we measured activation levels of the transcription factor NF-κB, a primary factor resulting in expression of NOS2. We found that NF-κB was slightly activated in cells treated solely with manganese or cytokines, whereas cells treated with both manganese and cytokines showed the highest levels of activation. Also, we found that these ligands function through an NF-κB dependent mechanism. Treatment of cDIM1 to astrocytes already treated with manganese and cytokines caused decreased activation of NF-κB, while addition of GW9662 to similarly treated cells resulted in increased activation of NF-κB. While these compounds were effective at manipulating induction of the nos2 gene, they had no effect on induction of guanosine tri-phosphate cyclohydrolase (GTPCH) the rate limiting enzyme for the production of tetrahydrobiopterin (BH4), a cofactor essential to the conversion of arginine to NO, Thus, these novel PPARγ ligands can influence manganese- and cytokine-induced production of NO by an NF-κB dependent mechanism.

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INTRODUCTION

Neurodegenerative diseases are a class of disorders that affect brain function by the deterioration of necessary neuronal pathways. They are divided into two groups: conditions which affect locomotion and those that affect memory and are associated with conditions of dementia. These diseases include, but are not limited to, Amylotropic Lateral Sclerosis, Multiple Sclerosis, Huntington's Diesase, and Parkinson's Disease.

Parkinson's disease (PD) is one of the leading causes of death by neurodegenerative diseases. Statistics have shown that it affects 1 in 625 people, with a mean age of 55 years, indicating increased risk in the elderly population (www.**cdc**.gov/nchs/data/factsheets/Parkinsons.pdf). Clinical cases usually number about 200 in 100,000 though approximately 2% of the population suffers from it at any given time (http://www.pdf.org/AboutPD/). It was first characterized by James Parkinson in 1817 who documented its symptoms, though the associated neuropathologies were not discovered until the 1960's. PD is part of a larger family of similar diseases known as parkinsonism.

The exact etiology of PD is not clear, though symptoms result from the destruction of dopaminergic neurons of the substantia nigra pars compacta. Lewy bodies, aggregations of the protein α-synuclein, are generally present, though their role is not well understood. Additional pathologies include neurofibrillary tangles (usually of the cerebral cortex) and increased activation of microglia. Symptoms vary among patients,

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This thesis follows the style and format of the Journal of Neuroscience.

but generally fall into two categories: those of a physical nature such as rigidity, problems with balance, and akinesia (lack of movement) and those of a psychological nature such as depression, dementia, and memory loss.

Several genetic abnormalities have been identified, each contributing to the disease's onset, though interplay among them isn't yet well understood. Current theories suggest genetic factors confer a vulnerability that can then be exploited by environmental toxins, including pesticides and metals. Among the more studied toxic agents are 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), paraquat, and rotenone (Kurkowska-Jastrzebska et al., 1999). The substances are commonly used models because they quickly induce symptoms seen in PD.

Much of our current knowledge of parkinsonism results from research using MPTP. The compound was first discovered when a group of drug addicts injected a batch of MPTP-tainted heroine. The addicts developed symptoms similar to those in seen in parkinsonism patients (Langston et al., 1983). It was later discovered that MPTP injection into rodents and primates recapitulated many of the neurologic and neuropathologic features of PD in humans, leading the hypothesis that environmental agents may play an important role in the disease process.

Manganese

Manganese is a divalent transition metal similar to iron in that it can be either necessary for vital function or toxic to it depending on the concentration ingested. Levels of manganese are not controlled by its sequestration, but rather through regulation of its

reabsorption and secretion, as well as by the blood brain barrier (Aschner and Aschner, 1991). Its removal from the brain is very slow (Dastur et al., 1971).

Manganese poisoning may occur in both industry and agriculture (Rodier, 1955). The resulting disorder, termed manganism, is characterized by neurologic symptoms resembling PD, including dystonia as a result of loss of striatal dopamine. While symptomatic similarities exist between parkinsonism and manganism, there are major differences both histological and physiological (Calne et al., 1994). Literature suggests that manganese wields toxic effects on mitochondrial function and Ca^{2+} homeostasis.

After manganese is administered in vivo, it accumulates within nuclei of the basal ganglia, including the globus pallidus, striatum, and substantia nigra pars reticulata (Yamada et al., 1986). Mitochondrial dysfunction is central to the neurotoxicity of Mn. Underlying this effect is the known capacity of Mn to affect mitochondrial calcium dynamics (Liu et al., 2006). Both calcium and manganese are taken into the mitchondria by the uniporter Ca^{2+} pump at normal physiological levels, but excessive Mn^{2+} will inhibit Na^{+}-dependent efflux of calcium, resulting in increased levels of matrix calcium (Konji et al., 1985). Manganese binds tightly to the inner mitochondrial membrane, where it is oxidized to M^{3+}, a species thought to inhibit oxidative phosphorylation (Gunter and Puskin, 1975). Overaccumulation of Mn^{2+} can interfere with oxidative phosphorylation by replacing Mg^{2+} or Ca^{2+} at binding sites of enzymes (Gunter and Pfeiffer, 1990). Astrocytes are able to avoid negative consequences by rerouting principal ATP production through glycolysis (Almeida et al., 2001). While Na^{+}-dependent efflux of Ca^{2+} is much faster than the Na^{+}-independent mechanism, Mn^{2+}

clearance does not happen in this manner. The amassing of Mn^{2+} in mitochondria aids in explaining its accumulation in the brain. Studies suggest that more Mn^{2+} promotes Ca^{2+} overload within the mitochondria, thereby enhancing neuronal apoptosis (Liu et al., 2005).

The pathophysiology of parkinsonism points to destruction of neurons in the substantia nigra pars compacta of the basal ganglia. The basal ganglia is a group of neurons whose primary responsibility is the partial regulation of voluntary movement. Even though there exists no direct connection with the spinal cord, the basal ganglia exerts its effects by receiving its input from the cerebral cortex and relaying information back to the cortex through the thalamus and brainstem. It consists of four nuclei, the striatum, the globus pallidus (pallidum), the substantia nigra, and the subthalamic nucleus. The striatum receives input from the cortex and then projects to the substantia nigra and the globus pallidus, which contains an inner and an outer segment. Together with the subthalamic nucleus, the inner pallidum comprises the output nuclei, which projects to both the thalamus and the brain stem (Albin et al., 1989).

The cortex sends excitatory glutamatergic signals to the striatum, which in turn relays it to other areas of basal ganglia. The vast majority of these cells are GABA-ergic and inhibitory by nature, including the two output nuclei which send constant inhibition to their targets in the brainstem and thalamus. There are two pathways that control this inhibition, the direct pathway and indirect pathway. The direct pathway travels from the putamen of the striatum to the inner pallidum and then onto output nuclei. Since these neurons are GABA-ergic, activation of the direct pathway inhibits the pallidal regions

which allows for the “de-repression” of neurons in the thalamus and brainstem, and thereby increases motor activity. The indirect pathway passes from the putamen to the external globus pallidus and then onto subthalamic nucleus before re-joining the direct pathway at the internal globus pallidus. Because the connection between the subthalamic nucleus and the internal pallidum is glutamatergic, activation of this pathway will increase the inhibition of the internal pallidum, which will then yield greater motor activity through the decreased inhibition of the thalamus and brain stem (Hoover and Strick, 1993). Dopamine is the neurotransmitter that activates both the indirect and direct pathways. Though they receive the same stimulus from the substantia nigra pars compacta, the pathways are able to exert differing effects because the dopamine is received by separate receptors, D1 to initiate the direct pathway and D2 receptors to initiate the indirect pathway (Gerfen et al., 1995).

Symptoms of Parkinson’s disease stem from depletion of dopamine in the striatum, which results from degeneration of dopaminergic neurons in the substantia nigra pars compacta. With dopamine unavailable, neither the direct nor indirect pathway can be stimulated and tonic inhibition of the thalamus and brainstem cannot take place, resulting in extraneous movement characteristic of the disease. Dopamine mimetics have proven successful in reducing symptoms, but effects are not permanent (Albin et al., 1995).

Manganism vs. Parkinsonism

Principal pathologies resulting from manganism include neuronal degeneration and glial activation. Neuronal destruction occurs principally in the globus pallidus and

the substantia nigra pars reticulata, whereas neuronal degeneration characteristic of parkinsonism usually occurs in the substantia nigra pars compacta (Yamada et al., 1986). Reactive astrogliosis is also a key feature (Bikashvili et al., 2001). As stated previously, neuronal degeneration of the pre-nigrostriatal neurons is seen in parkinsonism. However, it is post-synaptic destruction that is more common in manganese poisoning (Kessler et al., 2003).

Such neuronal destruction increases the cytoplasmic levels of Ca^{2+} to potentially toxic planes (Gavin et al., 1990). Increased matrix calcium leads to amplified levels of oxygen radicals, including Reactive Oxygen Species (ROS) (Kowaltowski et al., 1995). Mitochondrial dysfunction may then affect several downstream factors leading to transcription of pro-inflammatory genes, including inducible Nitric Oxide Synthase (iNOS). Increased manganese concentrations eventually lead to increased activation of NF-κB through the NIK pathway which leads to phosphorylation and activation of NF-κB (Barhoumi et al., 2004).

Nitric Oxide and the Inflammatory Response

Microglia are a type of glial cell discovered by del Rio-Hortega, a student of Ramon Y Cajal. They have many functions, including the regulation of neuronal differentiation and apoptosis. It has also been postulated that microglia can aid the growth and division of oligodentrocytes and astrocyes through the production of cytokines. Their primary function, however, is inflammatory. When brain injury occurs, microglia actually transform into cells with smaller processes and larger cell bodies. These microglia are then known as “activated” or “reactive” (Bikashvili et al., 2001). It

is important to distinguish between a single transient microglial response due to isolated occurrences and continual activation as it occurs during a disease state, such as Parkinson's Disease. Other studies indicate that neuronal cell loss is due to increased Reactive Oxygen Species (ROS). When compared to most other brain regions, the substantia nigra is replete with microglia (Kim et al., 2000b). Microglia are the principle immunological component of the nervous system. They are very reactive and take action quickly and continuously to injury within the central nervous system. Once the microglia sense damage, they respond to the site of injury and proliferate there (Stefano et al., 2004).

Production of NO from arginine in the body is catalyzed by a group of enzymes known as nitric oxide synthase(s). There exist three principle different subtypes which share approximately 50-60% homology (Masters et al., 1996). They are eNOS (endothelial NOS, also called NOS1), iNOS (inducible NOS, also called NOS2) and nNOS (neuronal NOS, also called NOS3). NOS1 and NOS3 are consititutive, and are activated by the presence of calcium and calmodulin. Upon an activation signal, NO is synthesized in very low concentrations by NOS1 and NOS3 and activates the second messenger cyclic GMP, which will then regulate a series of other signals. NOS2 is not present in resting cells, but rather is "turned on" by an immune signal, including infection in a number of cells like endothelium, monocytes, macrophages, and astrocytes. In contrast to the other two isoforms, it is independent of intracellular calcium levels (Xie et al., 1993). NOS exists in dimer form and this configuration is stabilized by the presence of the necessary co-factor tetrahydrabiopterin (BH4) (Tzeng et

al., 1995). Outside of its function in NOS stability, it is also thought to aid NOS mRNA stability (Stuehr, 1997). Its availability is rate limiting in NOS synthesis (Kidd et al., 2005).

NF-κB

NF-κB is a cytokine-inducible transcription factor key to the inflammatory response. It is made up of various complexes of the Rel family of proteins, which include p65 (RelA), RelB, c-Rel, p50, and p52. Common among them is a 300 residue domain (termed Rel homology domain) that is important for both dimerization and DNA binding. The NF-κB family of proteins exists in the cytoplasm in various combinations and remains there until activation. A myriad of factors, both environmental and endogenous, can trigger the NF-κB signaling pathway, which eventually results in the activation of inducible nitric oxide synthase (iNOS). Activation results from phosphorylation of two serine residues (Ser 32 and 36) of the inhibitory sub-unit, IκB. Its phosphorylation leads to its subsequent ubiquitination and degredation by the 26S proteasome. Ubiquitination of IκB must also be specific. In order for IκB to be degraded it must be ubiquitinated at Lys 21 and Lys 22. Site specific mutation (lysine to arginine) leads to a protein that is phosphorylated but not degraded, and therefore does not properly release NF-κB (Rodriguez et al., 1996).

NF-κB has many endogenous activators, including the pro-inflammatory cytokines. Studies in C6 glioma cells have shown that none of the pro-inflammatory cytokines (TNF-α , IL1—β, and IFN-γ) and LPS are unable to induce NF-κB activation alone, but only in combination with one another (Pahan et al., 1999; Uehara et al., 1999).

There is also a wide range of agents besides those mentioned can induce nuclear translocation and activation of NF-κB. It is of note that many of these inducers of NF-κB are also inducers of iNOS expression in certain cell types namely the β-amyloid protein present in Alzheimer's disease which has been shown to induce both NF-κB and iNOS in the mouse astroglial cell (Heneka et al., 2003).

TNF-α activates NF-κB by interacting with TNF-α receptor associated factors (TRAF) which then in turn activates NF-κB inducing kinase (NIK)(Kim et al., 2005; Yang et al., 2005). Other cytokines follow similar pathways. Activated NIK then activates the inhibitory κB complex which then phosphorylates serine 32 and 36 of IκB. NF-κB is then free to bind DNA (Barhoumi et al., 2004).

NF-κB dependent gene expression requires several co-transactivators that may function by modifying chromatin structure or by otherwise physically assisting transcription factor binding. The NF-κB subunit p65 requires the coactivator CBP (cyclic AMP response element being protein [CREB]-binding protein) and p300 (Gerritsen et al., 1997; Perkins et al., 1997). These coactivators are known to mediate histone acetyl-transferase (HAT) activity. HAT proteins place acetyl groups on the histones, thereby causing dissociation from the chromatin and allowing transcription to occur. Conversely, repressors often work through association with histone deacetylases (HDACs). As their name implies, they act through deactylation of histones and subsequent reassociation of histones with associated DNA sequences. N-CoR (nuclear receptor corepressor) and SMRT (silencing mediator for retinoid and thyroid receptors) are corepressors that act in the manner described above, in that they partner with HDACs

(specifically HDAC3) to repress transcription. They are known to do this by binding with a number of nuclear receptors and physically impeding transcription (Chen and Evans, 1995; Horlein et al., 1995; Glass and Rosenfeld, 2000; Guenther et al., 2000; Li et al., 2000; Zhang et al., 2002; Yoon et al., 2003). Interestingly, recent studies have shown that N-CoR and SMRT are also necessary for repression of NF-κB activated genes, including nos2 (Hoberg et al., 2004; Ogawa et al., 2004; Perissi et al., 2004).

The subunit p65 contains at least five serines that are phosphorylated at various times, though the functions of all phosphorylation events are not completely clear. However, it is likely that phosphorylation of serine 276 is necessary for coactivation (Okazaki et al., 2003). Similar to p65, the p50 subunit also requires coactivating factors. Both steroid receptor-coactivator-1 (SRC-1) and nuclear receptor coactivator-1 (NCoA-1) are necessary to insure proper DNA binding (Heery et al., 1997; Torchia et al., 1997; McInerney et al., 1998).

Earlier studies identified NF-κB as an important transcription factor in the LPS-induced expression of iNOS in the murine RAW 264.7 macrophage cell line. Deletional analysis of iNOS showed locations of NF-κB binding sites. Mutation of these sites exposed an inability of the transcription factor to bind and commence expression of the reporter gene (Kim et al., 1997).

Important differences have been identified in the regulation of iNOS expression in different cell types. For instance, in mouse macrophages, the downstream NF-κB site between -85 to -76 is apparently more important than the upstream NF-κB site at -971 to -962 for LPS inducibility. However, in TNF-α , IL1-β, and IFN-γ-stimulated vascular

smooth muscle, the upstream NF-κB site at -971 to -962 is more functional. Furthermore, both LPS induction and the synergistic response to LPS plus IFN-γ were mapped to an upstream enhancer DNA sequence that included the upstream NF-κB site at -971 to -962. Both the upstream and the downstream NF-κB sequence elements have been shown to bind various NF-κB family members in different cell types and in response to differing stimuli. Studies reveal that particular NF-κB proteins bind to different NF-κB sequences with distinct affinities in a cell type and stimulus dependent fashion (Lowenstein et al., 1993; Alley et al., 1995). The complete mechanism is not yet understood.

Gavrilyuk and colleagues show that the presence of the proximal NF-κB binding sites is not sufficient to confer induction by LPS and cytokines (Gavrilyuk et al., 2001). Instead, upstream activator sequences are needed. There are several response elements that appear cooperative in NF-κB binding, some of these include Oct-1 elements, interferon response elements, and JAK/STAT binding elements.

PPARγ

The peroxisome proliferator activated receptors (PPARs) are a group of nuclear receptors with a wide range of functions across cellular and molecular biology. Originally named for their ability to proliferate peroxisomes in rodent livers, they have been connected to functions as diverse as metabolism, inflammation, and cell differentiation. Three different isoforms, PPARα, PPARδ, and PPARγ have been identified. Each is transcribed by different genes and therefore has slightly different binding domains and binding partners. PPARα is found in the liver, heart and kidney.

PPARδ is expressed in most tissues and PPARγ is most commonly found in adipocytes and macrophages. The distinct distribution of tissues implies that each isoform has unique functions.

PPARγ is a member of the nuclear receptor family whose activity is regulated by direct binding of hormones, vitamins, and various lipid metabolites. Generally, once the ligand binds to PPARγ, it then heterodimerizes with Retinoid X Receptor-α (RXRα) before translocating to the nucleus and binding to specific DNA sequences known as PPAR response elements (PPREs) (Cullingford et al., 1998; Qi et al., 2000). It is through this classical pathway that it exerts its effects on adipogenesis and glucose homeostasis for which it is most well known. Binding to PPREs is known to induce adipogenesis as well as monocyte differentiation (Kim et al., 2000a). PPARγ is composed of several functional domains, and contains a highly conserved DNA binding domain and two transactivation domains: one transactivation domain, a N-terminal ligand independent domain and a C-terminal domain that is very much ligand dependent (Klein et al., 2005). This C-terminal domain is responsible for ligand binding, heterodimerization, and transactivation. PPRE binding, as is considered a positive regulatory event (Tanaka et al., 2005). When united with ligand, the ligand binding domains alter their conformation to allow recruitment of coactivators. This recruitment of coactivators depends upon a highly conserved motif that changes conformation to become part of the "ligand binding pocket" when ligand is attached (Hegele, 2005). While many coactivators have been characterized, the most commonly studied for PPARγ activation is SRC-1 which recruits

p300 and CREB-binding protein (CBP), though CBP binds directly to PPARγ, in some cases without the aid of SRC-1 (Qi et al., 2000).

Additionally, recent studies show PPARγ to be a major participant in inflammatory processes. Both natural and synthetic ligands have been known to exert anti-inflammatory effects in experimental cases of encephalomyelitis, rheumatoid arthritis, and inflammatory bowel disease (Kawahito et al., 2000; Diab et al., 2002; Adachi et al., 2006). Ricote and colleagues demonstrated that PPARγ is a negative regulator of inflammation in macrophages by inhibiting induction of iNOS, among other genes (Ricote et al., 1998). In contrast to its other functions in which it binds directly PPREs, PPARγ influences inflammation by imposing on inflammatory transcription factors such as NF-κB, STAT, and AP-1 (Chinetti et al., 1998).

Evidence has shown 15d-PGJ_2 to be an endogenous ligand for PPARγ. Thus it is believed that activation of PPARγ by 15d-PGJ_2 and other ligands may stimulate release of PPARγ from its repressor and therefore allow contact with co-activators and its successive nuclear translocation (Ide et al., 2003). It was recently shown that PPARγ activated by 15d-PGJ_2 competes with p300/CBP for transactivation of the IFN-γ promoter, thereby inactivating it (Farrajota et al., 2005). As stated previously, there is clear evidence that activation of PPARγ attenuates pro-inflammatory cytokine and iNOS expression in astrocytes (though not human brain macrophages) and, by extension, lowers nitrite production. Such anti-inflammatory effects are activated by structurally distinct ligands that include non-steroidal, anti-inflammatory drugs (NSAIDS), thiazolidinediones, and the aforementioned 15d-PGJ_2 (Consoli and Devangelio, 2005;

Townsend and Pratico, 2005; Zhao et al., 2006). Research shows that 15d-PGJ_2 inhibits NF-κB at multiple locations, both diminishing its capacity to bind DNA and hindering its ability to migrate to the nucleus by inhibiting phorphorylation of IKK (Zhao et al., 2004).

A great deal of recent research has focused on the thiazolidinediones. This class of PPARγ ligands, which includes troglitizone, rosiglitizone, and ciglizitone, is most well known for its anti-diabetic effects. Further investigation has shown clinical utility in other areas as well, including inflammation (Consoli and Devangelio, 2005). Recent evidence has shown that this class of pharmacological agents has diminishing effects on BH4 synthesis by inhibiting its rate limiting enzyme, guanonsine triphosphage cyclohydrolase (GTPCH) (Linscheid et al., 2003).

Recently, the mechanism PPARγ mediated inhibition of the NF-κB pathway has been established. Activation and nuclear transclocation of PPARγ leads to its association with complexes of NCoR and HDAC3 that are present near the promoters of inflammatory genes, such as nos2. Such association does not allow recruitment of necessary cofactors that are accountable for the elimination of the NCoR repressor and it remains bound to the promoter, disallowing transcription (Pascual et al., 2005).

A new class of PPARγ agonists has been characterized. 1,1-Bis (3'indolyl)-1-(p-substitutedphenyl methanes) also called diindolylmethanes or DIMs have shown efficacy in retarding growth within breast, colon, and pancreatic cancer cell lines by activation of PPARγ. It has been associated with cell division associated pathways, including inhibition of the G_0/G_1 –S phase the cell cycle (Chintharlapalli et al., 2004; Hong et al.,

2004; Qin et al., 2004). To date, however, these compounds have not been tested for their efficacy in anti-inflammatory capacities. We will attempt to answer this question by first establishing a relationship between these ligands and affected levels of produced NO. We will then seek to establish the mechanism of action of these ligands in part, by determining their relationship with NF-κB. We will further seek to determine the mechanism of this interaction by testing these agent's abilities to diminish NO synthesis by inhibiting production of BH4's rate limiting enzyme, GTPCH.

MATERIALS AND METHODS

Cell Culture

Primary cortical astrocytes were isolated from neonatal mice pups and plated on 60 mm plates obtained from VWR (West Chester, PA). Cells were incubated in CO_2 tanks in Minumum Essential Media obtained from Invitrogen, with 10% Fetal Bovine Serum and a combination of Penicillin, Streptomycin, and Neomycin, all obtained from VWR. Mice containing a nos2 knockout were obtained from The Jackson Laboratory (Bar Harbor, ME) and were cultured in identical manner.

Fluorescence Microscopy

Nitric Oxide was measured using the fluorescent marker, 4-amino-5-methylamino -2',7'-difluoroflorescein diacetate (DAF-FM) obtained from Molecular Probes (Carlsbad, CA), prepared as a 5mM stock solution in DMSO and diluted in culture media to a final concentration of 5μM for imaging studies. For analysis of nitric oxide production in astrocytes, including both wildtype and astrocytes in which the nos2 gene has been excised, the fluorescence emission intensity of DAF-FM was determined kinetically by collecting images at $488nm_{EX}/515nm_{EX}$ emission at five minute intervals for 55 minutes at exposure times of 20 milliseconds in order to allow the reaction of NO with DAF-FM to reach equilibrium. After incubation with various treatment media, cells were treated and incubated with DAF-FM for a period of ten minutes. Intensity data were then analyzed by calculating a normalized fluorescence value for each image as df/F_0, where df represents the background-subtracted fluorescence of a given cell at time

(t) divided by the fluorescence of the same cell at time zero. Analysis was performed using Slidebook, v. 4.1; Intelligent Imaging Innovations, Inc. (Denver, CO). Data were further analyzed by averaging the maximum intensities and comparing to maximum averaged control values, and displayed as fold difference. Furthermore, sequential fluorescence time points for each image, again calculated as df/F_0, were displayed as line graphs.

NF-κB Activation

To determine the amount of NF-κB activation elicited, we obtained a transgenic mouse from the laboratory of Dr. David Brenner that contained astrocytes in which a novel enhanced green fluorescent protein (EGFP) reporter had been placed. This reporter gene is under the control of NF-κB and fluoresces as NF-κB is activated and induces transcription. Qualitative images were taken in the presence of manganese, cytokines, and PPARγ ligands to determine their role in NF-κB activation. These cells were cultured in the manner described above.

Real-Time

Astrocytes were isolated from mice. RNA was isolated from astrocytes plated on 60 mm tissue culture plates from VWR (West Chester, PA) using the RNeasy Mini Kit from Qiagen (Valencia, CA) . Previous to isolation, astrocytes were treated for 24 hours with combinations of 50 μM $MnCl_2$, pro-inflammatory cytokines Tumor Necrosis Factor-α (TNF-α) and Interferon-γ (IFN-γ), as well as varying concentrations of the peroxisome proliferator activated receptor-γ (PPAR-γ) agonist 1,1-Bis (3'-indolyl)-1-(p-trifluormethylphenyl) methane (cDIM1) and antagonist GW 09266. After isolation,

RNA was quantified using a Beckman-Coulter Spectrophotometer. The reverse transcription was carried out with 1μg of RNA, 1 μL of dNTPS and 1 μL of oligo DTs, both from Bio-Rad (Hercules, CA). Mixtures were then incubated at 65°C for five minutes, before being briefly chilled on ice. A second mix was created using 5X 1st strand buffer, .1 DTT, RNase Out RNase inhibitor, and Superscipt III Reverse Trancscriptase. Mixes were combined for each treatment and then incubated at 42°C for 50 minutes, then incubated at 70°C for 15 minutes.

Upon removal from the incubation, samples were brought up to 50 μL with DNase/RNase free water from Qiagen. 5 μL of sample was added to 20 μL of supermix which included 12.5 μL Bio-Rad's Sybr Green, 1.25 μL of both sense and anti-sense primers, and 5 μL of RNase/DNase free water. Primers were designed using the Beacon designer program. Samples were then spun down and briefly placed on ice. The plate was then loaded into Biorad's I-cycler. Samples were incubated in the I-cycler at 95°C for 3 minutes and 30 seconds, followed by alternating incubations of 10 seconds at 95°C and 1 minute at 55°C. They were then incubated at 55°C for 80 cycles of 1 minute. Melt Curves were also run to determine purity. Data was analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method outlined by Livak and Scmittgen (Livak and Schmittgen, 2001).

RESULTS

Nitric Oxide Production in Primary Astrocytes

We investigated whether manganese, together with cytokine activation of inflammation regulatory pathways, induces nitric oxide synthase (nos2) activity in mouse astrocytes. Nitric oxide synthase activity in primary cultures of astrocytes was estimated using an NO production indicator, difluoroflorescein diacetate (DAF-FM), and fluorescence microscopy (Figure 1). Manganese treatment of primary astrocytes increased nitric oxide production in a time-dependent fashion. Exposure of astrocytes to 50μM $MnCl_2$ and the pro-inflammatory cytokines 1μg/ml TNF-α and 10 ng/ml INF-γ did not significantly increase nitric oxide production at 0, 4, and 8 hours over saline control, but did increase nitric oxide production by 25% over controls following a 24h treatment (Figure 2). Based on these results, astrocytes were treated with either manganese or the aforementioned cytokines for 24h in all subsequent experiments. Addition of either $MnCl_2$ alone or the two cytokines in concert increased nitric oxide production 15 and 18%, respectively (Figure 3). Again, in these experiments, exposure to manganese and cytokines together elicited a synergistic increase in NO production greater than 35% (Figure 3).

Identical DAF-FM measurements were conducted in primary astrocytes cultured from knock out mice in which the inducible nitric oxide synthase gene (nos2) was deleted. In these nos2 null astrocytes, treatment with manganese and cytokines, both separately and together, had no effect on nitric oxide production (Figure 4). Thus, the

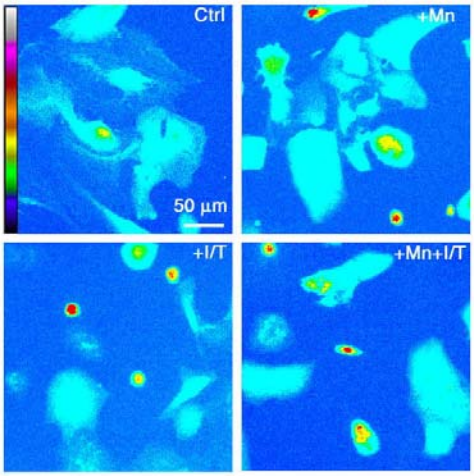


Figure 1 Synergistic enhancement of manganese and cytokines on nitric oxide production. Representative psuedocolored images of primary cultures of astrocytes. Astrocytes were treated with manganese, cytokines, and a combination of the two. Cells were visualized by fluorescence microscopy using cell permeant NO dye DAF-FM. The color bar represents differing levels of NO produced.

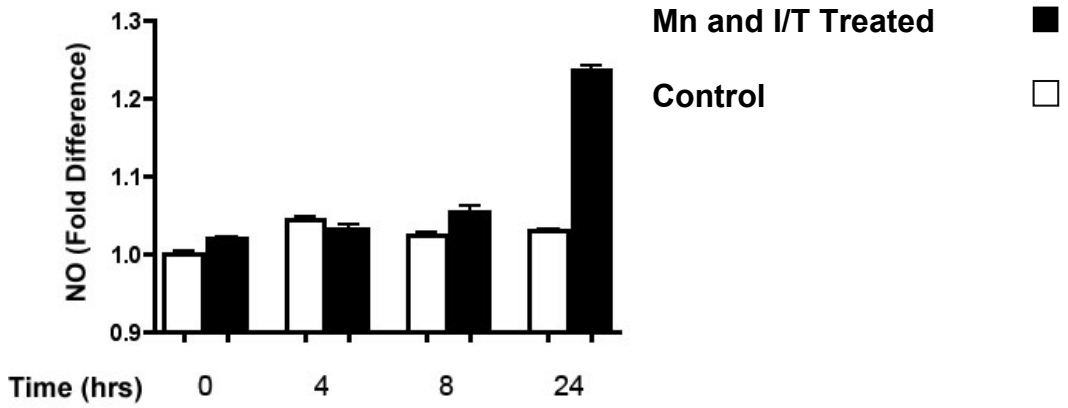


Figure 2 Time dependent production of nitric oxide in primary astrocytes. Cells were visualized by fluorescence microscopy using cell permeant NO dye DAF-FM. Bars represent percentage difference from control at 0 hrs (mean ± SEM of data compiled from 2 experiments; n=439). Significant difference ($p<0.05$) is indicated by different letters. Line graphs represent increase in df/Fo.

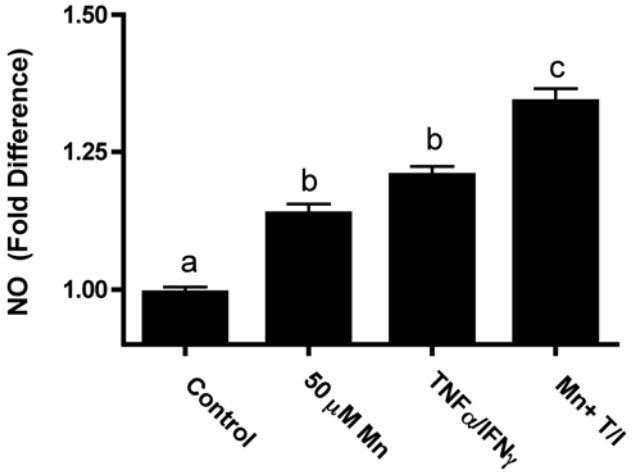


Figure 3 Synergistic enhancement of manganese and cytokines on nitric oxide production. NO production was measured by fluorescence imaging primary astrocyte cultures. Cells were visualized by fluorescence microscopy using cell permeant NO dye DAF-FM. Bars represent percentage difference from control (mean ± SEM of data compiled from 3 experiments; n=368). Significant difference ($p<0.05$) is indicated by different letters. Line graphs represent increase in df/Fo.

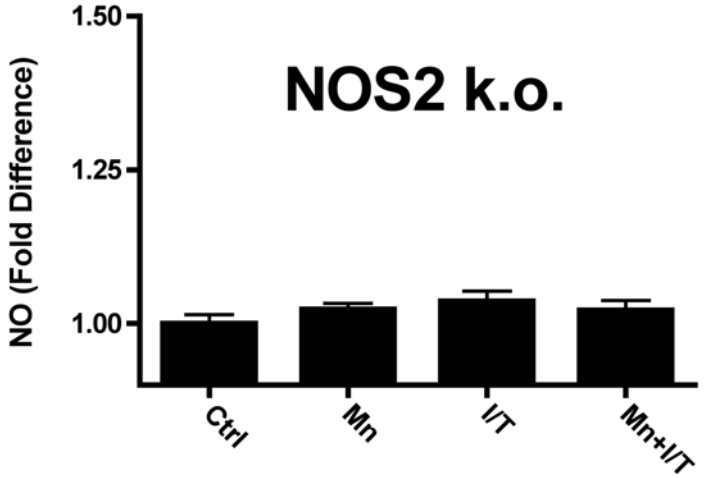


Figure 4 NO production in the presence of manganese and cytokines in *nos*2 knockout astrocytes (mean ± SEM of data compiled from 3 experiments; n=146). Cells were treated with manganese and cytokines. NO production was measured by fluorescence imaging primary astrocyte cultures using the cell permeant NO dye DAF-FM. Bar graphs represent increase in df/Fo.

increases in NO production, as well as well as their synergistic effects, are entirely due to the induction of nos2.

To investigate the role of peroxisome proliferator activated receptor-γ Mn/cytokine induction of nos2 activity, we used pharmacological manipulation to enhance or suppress PPARγ activity. The PPARγ antagonist GW9662 was added to astrocyte cultures as DAF-FM measurements of NO production were performed (Figure 5a). Treatment with both manganese and cytokines, in the presence of increasing concentrations of GW9662, caused a dose-dependent increase in NO production (Figure 5b). Addition of 0.1 μM GW9662 induced an increase of approximately 10%, in comparison to treatment with Mn/cytokines alone. Following a 10 μM GW9662 treatment, a greater than 35% enhancement of nos2 activity, as determined by nitric oxide production, was measured. The PPARγ agonist cDIM1 attenuated NO production in primary astrocytes in a dose-dependent fashion (Figure 6). Addition of the agonist at 0.01 μM and 1 μM significantly decreased NO production by approximately 20% and 28%, respectively. Thus, in primary astrocyte cultures, agonist-mediated activation of PPARγ suppressed nos2 activity; whereas, antagonists of PPARγ enhanced NO production in these glial cells. evels over the hour time course of the DAF-mediated NO measurement following treatment

In the hour following 24h of exposure to either $MnCl_2$ alone or the two cytokines without $MnCl_2$, NO increased steadily to its max value with both manganese and

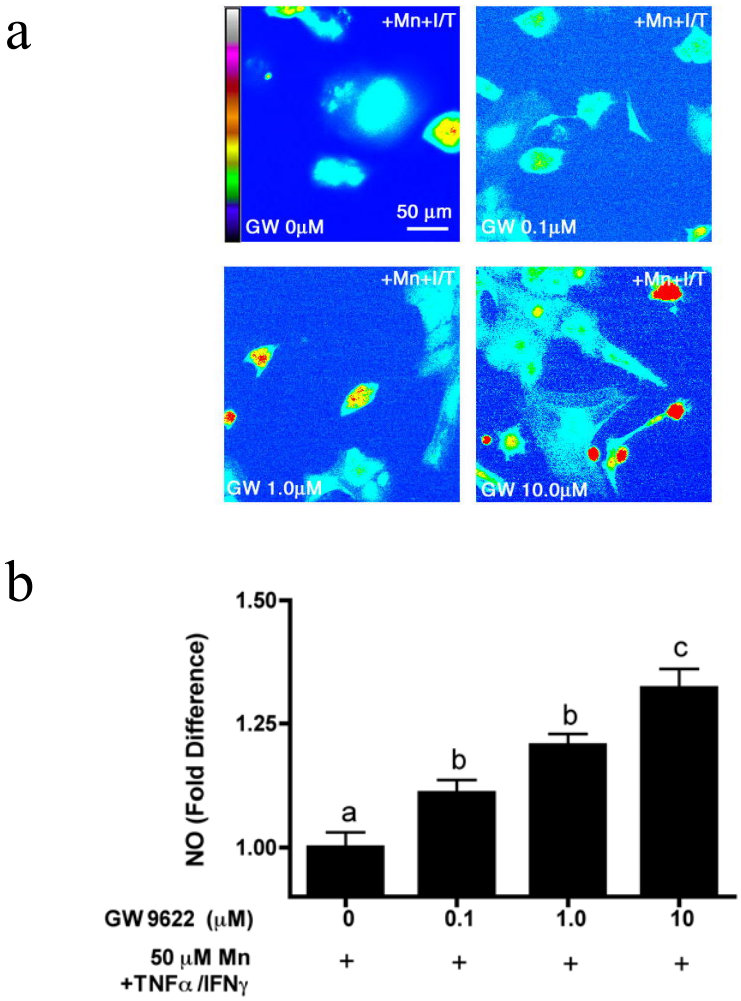


Figure 5 Effect of PPARγ antagonist GW 9662 on manganese- and cytokine- derived nitric oxide production in primary astrocytes. Cells were visualized by fluorescence microscopy using cell permeant NO dye DAF-FM. a) Representative psuedocolored images are shown of astrocytes that have been treated with manganese, cytokines, and the antagonist GW9662. b) Bar graphs represent increase in df/Fo (mean ± SEM of data compiled from 3 experiments; n=273). Significant difference ($p<0.05$) is indicated by different letters.

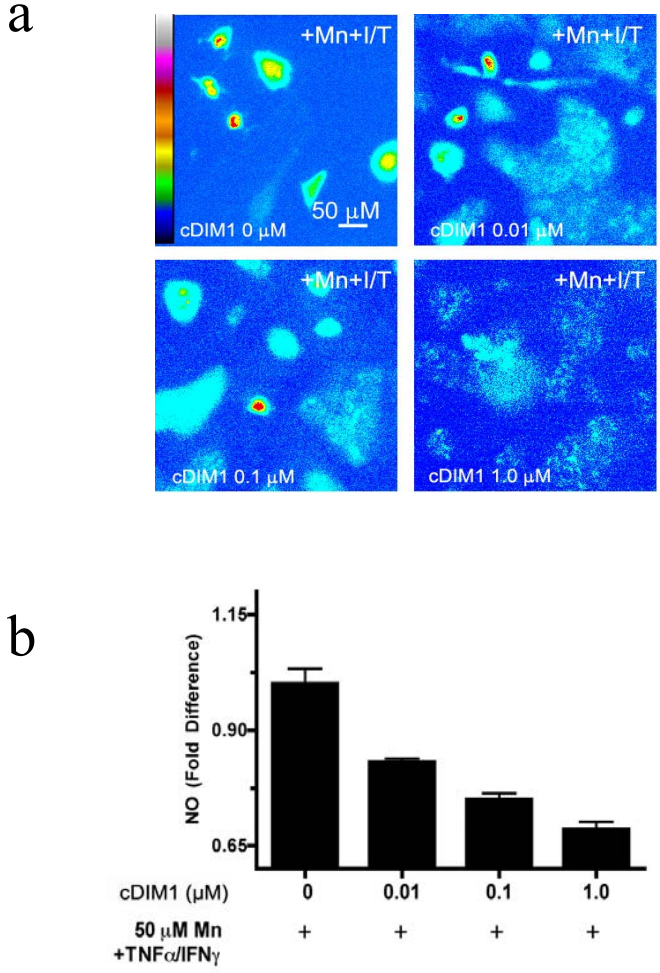


Figure 6 Effect of PPARγ agonist 1,1-Bis(3'-indolyl)-1-(p-trifluormethylphenyl) methane (cDIM1) on manganese- and cytokine-derived nitric oxide production in primary astrocytes. NO was visualized by fluorescence microscopy using a the cell permeant NO marker, DAF-FM. a) Representative psuedocolored images b) Bars represent percentage difference from control (mean ± SEM of data compiled from 3 experiments; n=244). Significant difference ($p<0.05$) is indicated by different letters.

cytokines. Addition of the PPARγ ligands, both agonist and antagonist caused a steady (though at times erratic) decrease (25%) and increase (28%) respectively over the hour of treatment when compared to the astrocytes treated only with manganese and cytokines (Figure 7).

NF-κB Activation in Primary Astrocytes

Having determined the efficacy of these PPARγ ligands to affect nos2 derived NO production, we sought to elucidate the mechanism underlying these changes. As stated previously, research has shown that PPARγ exerts it effects on inflammatory processes through interaction with NF-κB, a primary inflammatory transcription factor. To determine if PPARγ ligands function in an NF-κB-dependent manner, astrocytes with an enhanced green fluorescent protein (EGFP) reporter of NF-κB transcriptional activation were treated under various conditions and assessed for fluorescent activity (Figure 8). Thus, greater fluorescence corresponded to greater transcriptional activation by NF-κB. Under identical treatment conditions as described earlier, we added $MnCl_2$ and TNF-α and INF-γ both separately and in concert. We found that while the additions of cytokines and manganese both enhanced fluorescence over control cultures, the addition of the two in concert caused activation greater than addition of either by itself (Figure 9). Therefore, we see that manganese has an enhancing effect on cytokine-elevated, NF-κB-dependent transcriptional activation.

Furthermore, we sought to test the effect of PPARγ ligands on NF-κB-dependent transcriptional activation by adding both the PPARγ agonist cDIM1 and the PPARγ antagonist GW 9662 to cultured astrocytes treatments. We found that addition of the

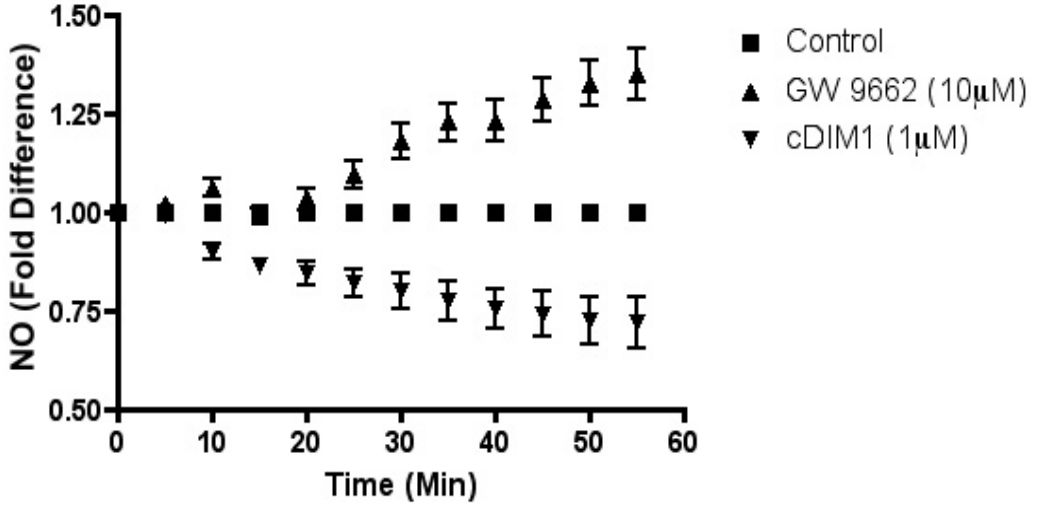


Figure 7 Time course of nitric oxide production in primary astrocytes treated with cDIM1 and GW9622. The control group was treated with manganese and cytokines while the treated group received manganese, cytokines, and PPARγ ligands. Lines represent percentage difference from manganese and cytokine treated controls over the same hour. (mean ± SEM of data compiled from 2 experiments; n=127).

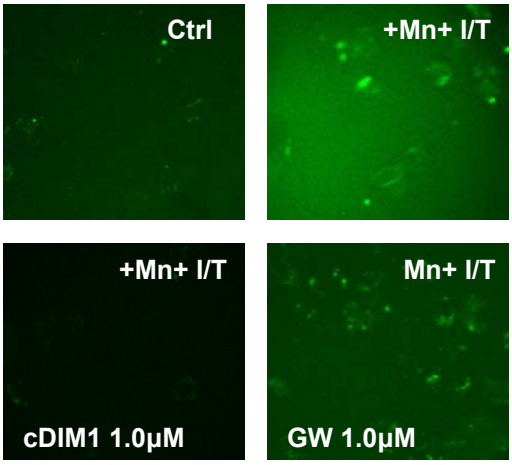


Figure 8 Effect of manganese and cytokines separately and together and with additions of cDIM1 and GW 9662 on NF-κB activation in primary astrocytes. Astrocytes contain an EGFP reporter that fluoresces green with increased transcriptional activation by NF-κB. Representative images are shown.

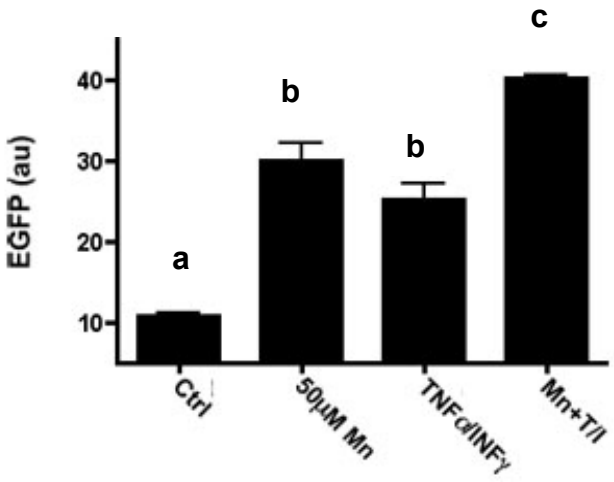


Figure 9 Effect of manganese and pro-inflammatory cytokines on NF-κB activation in primary astrocytes. a) Astrocytes contain an EGFP reporter that fluoresces green with increased transcriptional activation by NF-κB. b) Bar graphs are measures of fluorescence shown in arbitrary units (au), with n=5. Significant difference ($p<0.05$) is indicated by different letters, as shown by a, b, and c.

agonist diminished EGFP fluorescence in a dose dependent manner, with addition of 1 μM agonist causing the greatest degree in fluorescence and additions of 0.1 μM and 0.01 μM causing subsequently less inhibition (Figure 10). Suprisingly, however, it was noted that the greatest activation of EGFP fluorescence by the PPARγ antagonist did not occur with the highest concentration (10μM), but rather by addition of 1μM GW 9662. It

It should be noted that the 10μM treatment did induce activity, but to a lesser degree (Figure 11). Statistically, we found that such findings show that not only do these PPARγ ligands affect nitric oxide production by altering nos2 activation, they also do so by influencing the degree to which NF-κB is activated and therefore manipulate its ability bind to the nos2 promoter and activate its transcription.

Induction of GTPCH and nos2

With the understanding that PPARγ-induced alteration of NO production in primary astrocytes is NF-κB dependent, we inquired to know whether other mechanisms played a role in these variations. Specifically, we examined the effect of PPARγ on iNOS's necessary cofactor, tetrahydrobiopterin by monitoring the expression of its rate limiting enzyme, guanosine triphosphate cyclohydrolase (GTPCH). Consistent with our previous results, we found that manganese- and cytokine-treated astrocytes increased iNOS expression increased significantly, by almost two and half times (Figure 12a). Furthermore, GTPCH expression was significantly augmented by the addition of manganese and cytokines, specifically an increase of 120%. (Figure 12b). Also consistent with our previous findings, cDIM1 decreased iNOS expression by 80% (Figure 13a), while addition of GW9662 showed a corresponding increase of almost four

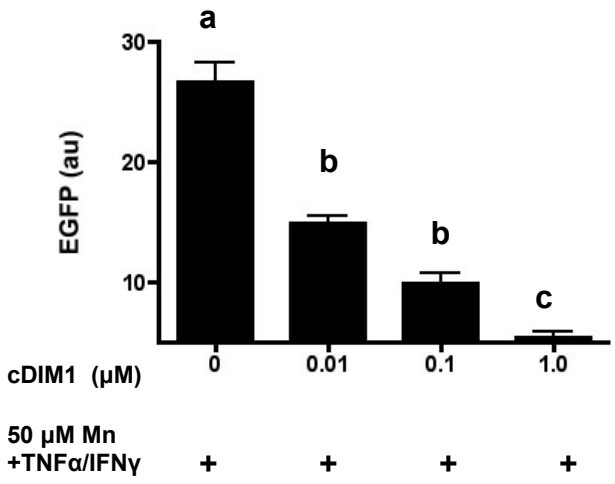


Figure 10 Effect of PPARγ agonist cDIM1 on manganese- and cytokine-derived NF-κB activation in primary astrocytes. Astrocytes contain an EGFP reporter that fluoresces green with increased transcriptional activation by NF-κB. Bar graphs are measures of fluorescence shown in arbitrary units (au), with n=5. Significant difference ($p<0.05$) is indicated by different letters, as shown by a, b, and c.

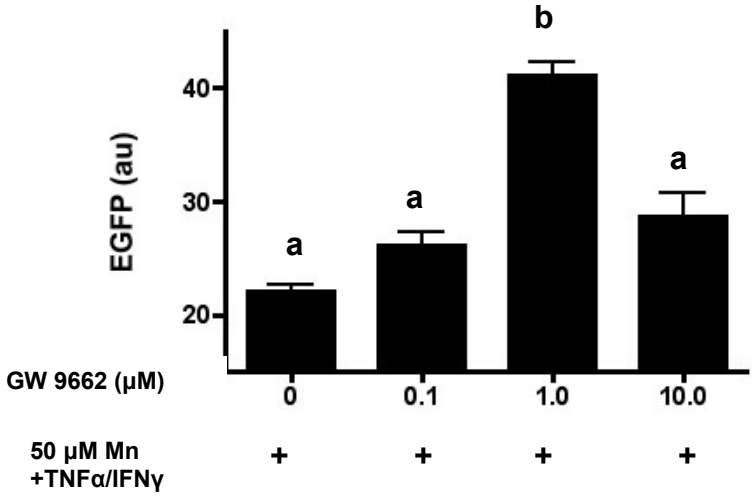
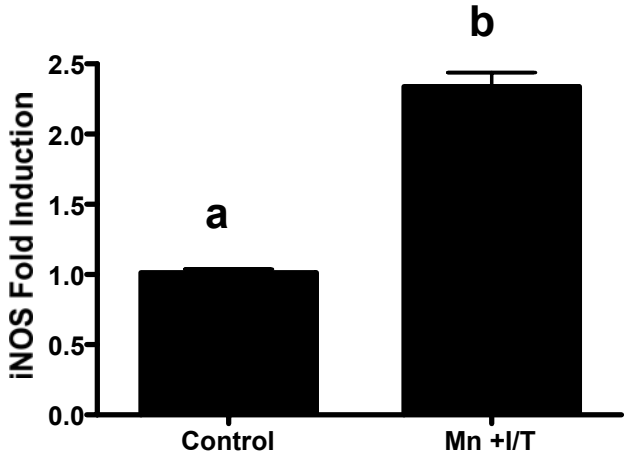


Figure 11 Effect of PPARγ antagonist GW 9662 on manganese- and cytokine-derived NF-κB activation in primary astrocytes. Astrocytes contain an EGFP reporter that fluoresces green with increased transcriptional activation by NF-κB. b) Bar graphs are measures of fluorescence shown in arbitrary units (au), with n=5. Significant difference ($p<0.05$) is indicated by different letters, as shown by a and b.

a



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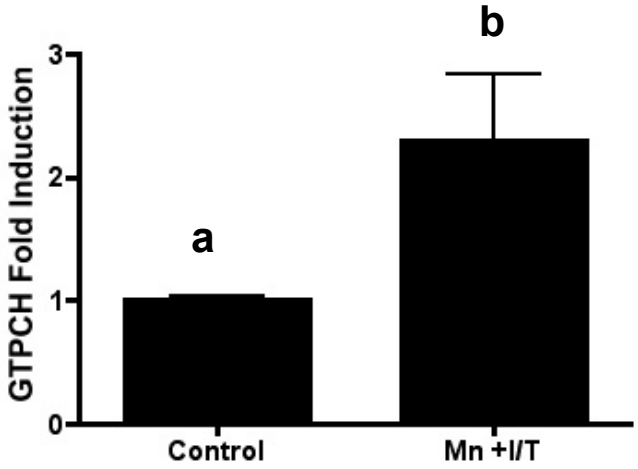
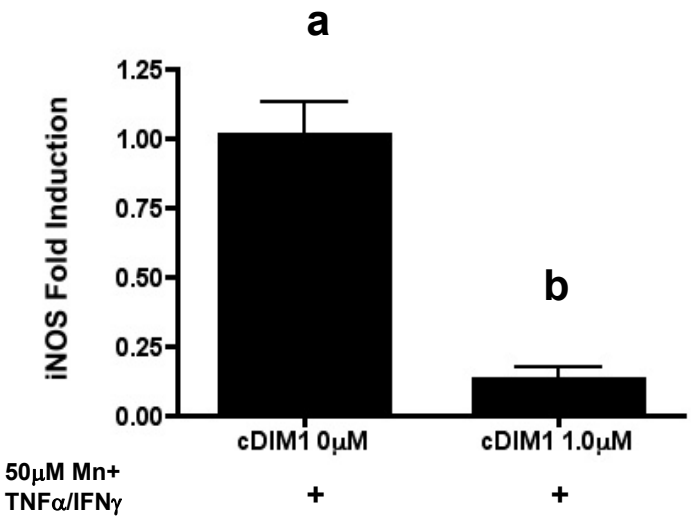


Figure 12 Effect of manganese and cytokines on induction of iNOS and GTPCH in primary astrocytes. a) Treatment effects on iNOS induction. b) Treatment effects on GTPCH induction. Expression was measured by real-time polymerase chain reaction and displayed as a percentage of the control with n=3. Significant difference ($p<0.05$) is indicated by different letters, as shown by a and b.

a



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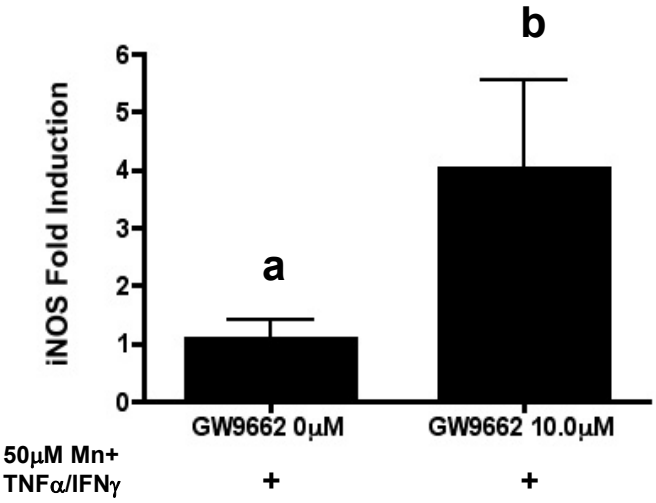

Figure 13 Effect of PPARγ ligands on iNOS induction in primary astrocytes. a) iNOS induction in the presence of cDIM1. b) iNOS induction in the presence of GW 9662. Expression was measured by real-time polymerase chain reaction and displayed as a percentage of the control with n=3. Significant difference ($p<0.05$) is indicated by different letters, as shown by a and b.

fold (Figure 13b). However, these ligands, both agonist and antagonist, appeared to have no effect on expression of GTPCH. There was no significant increase or decrease in GTPCH from control levels when treated with both the PPARγ agonist and PPARγ antagonist (Figure 14). Thus, PPARγ-induced manipulation of NO production is NF-κB dependent, but does not effect tetrahydrobiopterin or GTPCH levels.

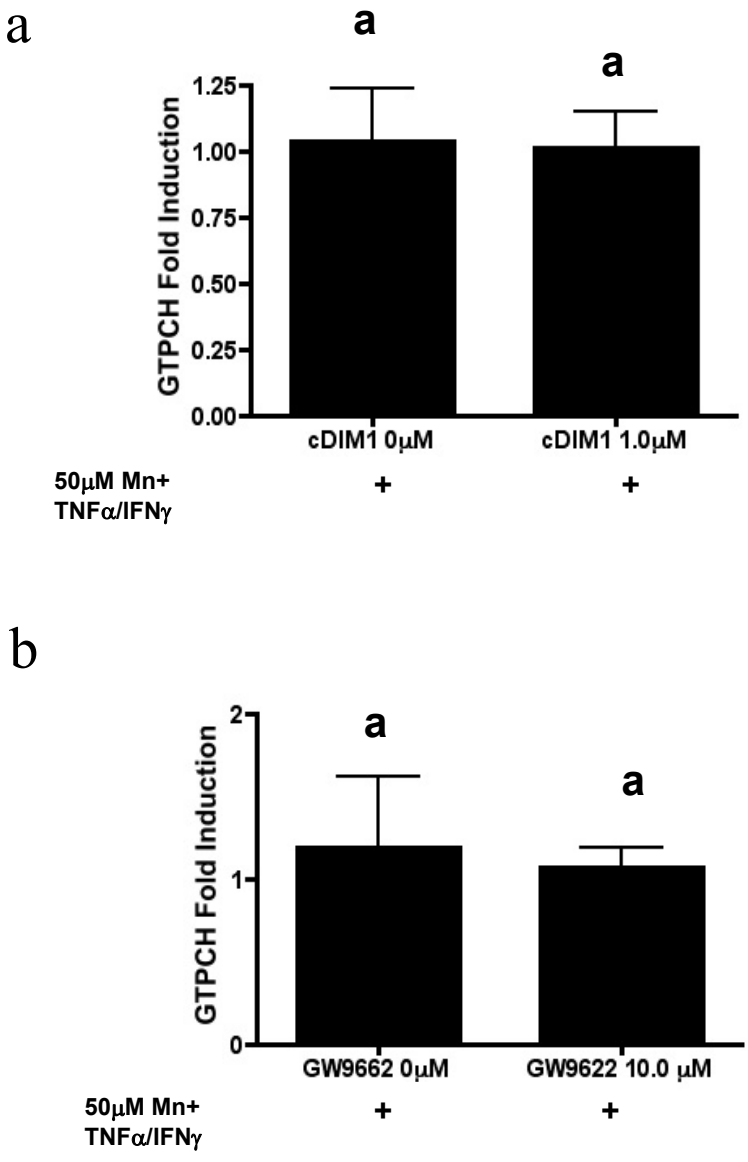

Figure 14 Effect of PPARγ ligands on GTPCH induction in primary astrocytes. a) GTPCH induction in the presence of cDIM1. b) GTPCH induction in the presence of GW 9662. Expression was measured by real-time polymerase chain reaction and displayed as a percentage of the control with n=3. Significant difference ($p<0.05$) is indicated by different letters, as shown by a.

SUMMARY AND CONCLUSIONS

Pathogenesis of glial inflammatory processes is a primary cause for parkinsonian disorders. Although the exact mechanism for many of these disorders has yet to be elucidated, research has indicated that nitric oxide is involved in these pathologies, specifically Parkinson's Disease (PD). Furthermore, it has been shown that several compounds including manganese can lead to increased NO production and subsequent damage to dopaminergic neurons, the primary cell type lost in PD.

Recent studies have clarified the mechanism by which Mn accumulation increases catalytic conversion of arginine to NO. Accumulation of manganese occurs within the inner mitochondrial membrane of glial cells. This buildup over a 24 hour period leads to marked increases in NO production by the astrocytes. This enhanced NO production stems from manganese-driven disruption of Na^+ pumps which in turn causes an accumulation of mitochrondrial calcium. The increase of mitochondrial calcium is toxic to the organelle and induces formation of reactive oxygen species (ROS), as well as decreased mitochondrial function(Liu et al., 2002; Barhoumi et al., 2004). Previously published reports show that increased ROS leads to increased NF-κB activation and therefore to induction of nos2. This study confirms reports of a synergistic increase in NO production and furthermore reports that this increase is due to transcriptional activation of nos2.

PPARs are ubiquitous compounds with several known capacities, including inducing transcription. The sub-type PPARγ is known to exert inhibitory effects on NO production. As such, we studied the ability of novel PPARγ effectors to alter the level of

manganese and cytokine induced glial NO. Previously these compounds had been used to suppress tumor growth, yet their efficacy in interdicting NO production had not yet been tested. We found that the PPARγ agonist cDIM1 attenuated NO production in cells previously treated with Mn and cytokines in a dose-dependent manner. Conversely, treatment of these cells with the PPARγ antagonist GW9662 led to a dose-dependent increase of NO production. Our findings indicate the effects of the agonist are not non-specific, (i.e. that it does act by pharmacologically manipulating PPARγ rather than by some non-specific effect.) They also suggest a baseline level of PPARγ inhibition, even under non-treated conditions.

Increased manganese treatment has been linked to increased activation of Nik, and connections between Nik and ROS have been suggested, though no evidence has been put forth. Nik is known to phosphorylate both isoforms of IκB Kinase (IKK), which in turn leads to the phosphorylation of IκB (the inhibitory sub-unit of NF-κB), its subsequent dissociation from the p65/p50 complex, and its ubquitination and degradation. The p65/p50 complex is then free from inhibitory signals and can cross the nuclear membrane bind to its promoter. It is thought that the signal created by mitochondrial accumulation of manganese and the signaling cascade produced by the pro-inflammatory cytokines converge upon NF- κB, together causing a greater activation and significant increase in NOS levels and NO production (Figure 15).

To determine whether or not manganese and cytokine treatment enhances NF-κB dependent transcriptional activation, and likely nos2 activation, we examined NF-κB EGFP reporter cells from transgenic mice. We found that addition of manganese and

cytokines partially activated NF-κB, while addition of the two in concert led to much stronger fluorescent activity (Figure 7). This is consistent with the idea that the signals are convergent upon NF-κB. Moreover, attenuation of EGFP signal displayed with the addition of the PPARγ agonist as well as the augmentation of signal with the addition of the PPARγ antagonist are strong indicators that these ligands operate through an NF-κB dependent mechanism. Such findings point to the possibility of pharmacological intervention as a method of interdicting manganese stimulated nitric oxide and by extension contributing to the possible prevention of parkinsonian disorders, most notably idiopathic PD (Figure 15).

This idea of synergistic enhancement provides rationale for studying the effects of both Mn and cytokines on endogenous NO production. It also mimics a possible clinical condition in which excessive accumulation of Mn causes an increase in the baseline level of NO produced. When an inflammatory event activates the cytokine pathway, the signal cascade combines with manganese-induced signals to create a condition where neuronal destruction occurs due to extended excessive NO exposure. Indeed, our study confirmed previous results that show that the combination of manganese and cytokines produces greater NO than either substance alone. Furthermore, it shows that while this increase is also reflected in increased induction of GTPCH, PPARγ influences this NO production solely through an NF-κB mechanism and not through the alteration of GTPCH.

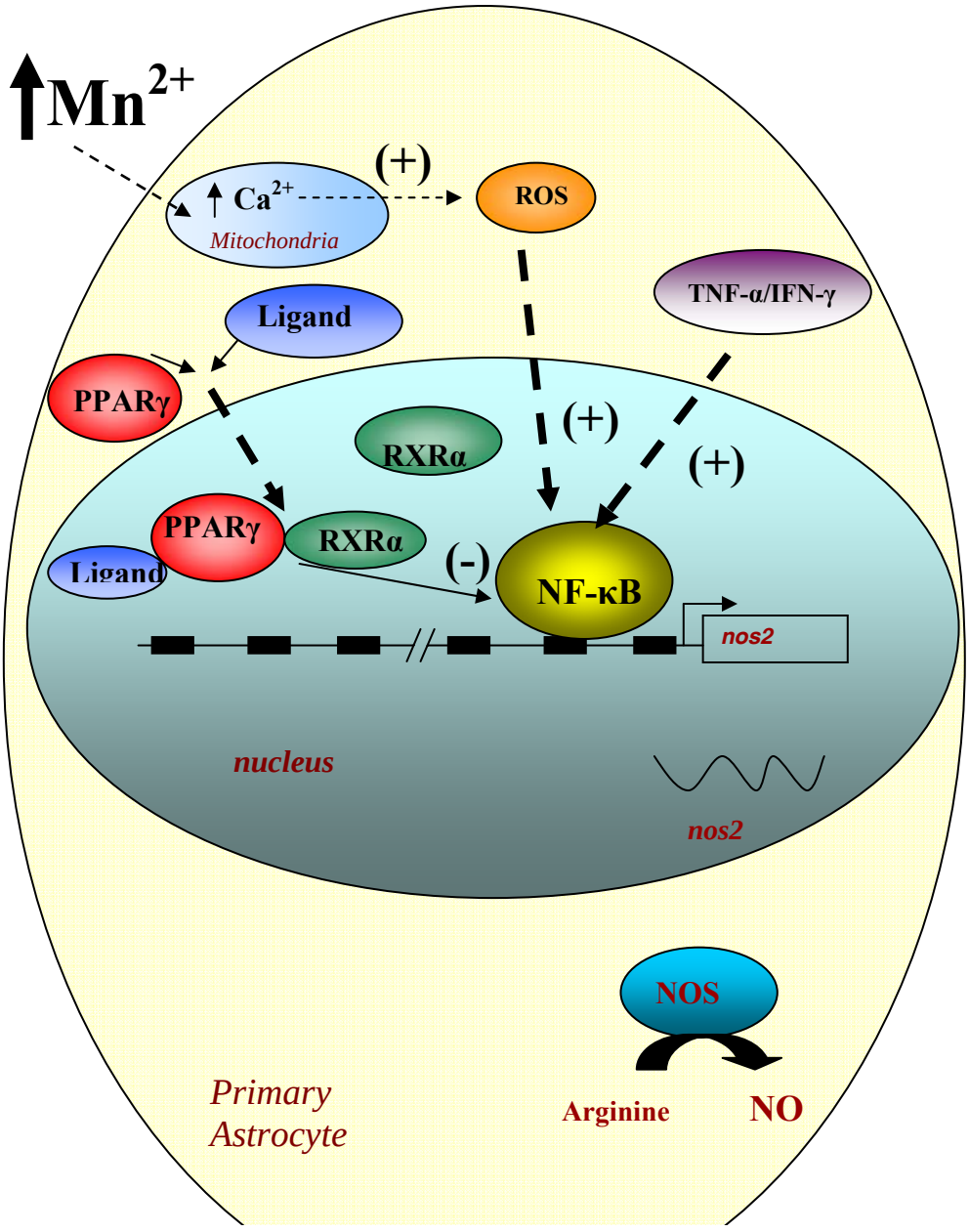

Figure 15 Exposure to manganese and cytokines causes a synergistic enhancement of NF-κB activation and subsequent production of NOS2 and increase in NO levels. This pathway may be inhibited by activation of PPARγ which inhibits NO production through an NF-κB mediated process.

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